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TITLE: *Inhibition of the Androgen Receptor Amino-Terminal Domain by a Small Molecule as Treatment for Castrate-Resistant Prostate Cancer*

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Purpose: The hypothesis of this study is that EPI-001 that targets the AR NTD will inhibit AR-driven recurrence of prostate cancer resistant to current methods of androgen deprivation or blockade.
Scope: Aim 1 will determine the impact of EPI-001 on castration sensitive tumor regression and re-growth in LuCap xenografts and on growth of their castration resistant forms. **Aim 2** will examine the impact of EPI-001 on castration sensitive and castration resistant growth of tumors with differing tumor androgen levels and differing ratios of ARv567es to full-length AR. **Aim 3** will elucidate the specific molecular mechanisms by which EPI-001 inhibits the activity of full-length AR and truncated ARv567es variants using in vitro models.
Progress: Tasks 1 and 3:We have completed the EPI-002 treatment in 5 xenograft lines in the second year of this study. These were done following castration and in castrate resistant growth states. Tasks 4 and 5: We have measured intratumoral androgen and found that they have a major impact on EPI-002 response. Task 1 and 3, we have developed new monoclonal antibodies during this past reporting period that will now permit specific assessment of AR-variant protein in tissues in response to EPI-002. In Task 6 we have shown during this past year that the transcriptome generated by the AR-Vs and inhibited by EPI is a complex of hetero and homo-dimers of AR-Vs and AR-FL receptors
Findings: AR-Vs signal by chromatin looping in the absence of androgens driven by the N-terminus- target of EPI.
Significance: The new antibodies to AR-Vs enable a more clear picture of AR-V EPI interaction on chromatin. Based on data so far in this program IND for EPI should be submitted in early 2015

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Introduction: During the past year data has been published from our group as well as others that the AR- splice variants are at least markers of resistance to enzalutamide and abiraterone when found in circulating tumor cells and are harbingers of a more lethal disease. Thus the need to develop and understand the mechanisms of action of EPI-compounds on the constitutively active AR spice variants is urgent. Furthermore, in this years report we will show new information on the mechanism of action of EPI compounds and how they effect AR-V interaction with chromatin. Finally, we will show the significance of newly developed specific AR-V antibodies and how they will be used to further dissect the mechanisms of action of the EPI compounds.

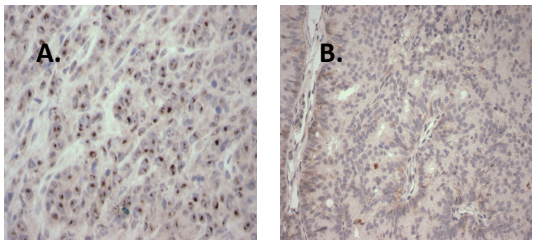


Figure 1. AR-V7 IHC in LuCaP 23.1 that expresses V7 (A) and LuCaP 35 human prostate xenograft that does not express V7 (B).

Accomplishments:

Aims 1 and 2 . Last year we reported the results of treatment of LuCap xenografts with EPI -001 as well as some of the responses in gene expression. During this past year of funding we have generated polyclonal and monoclonal antibodies that specifically recognize AR-V7 and AR^{v567es} on IHC as well as specifically on Westerns and IPs, **Figure 1 .** Since these antibodies have just become available we have asked for a NCE of the current grant in order to perform IHC on the EPI-treated xenografts and analyze the results. Including gene expression. This is especially important because as shown in **Figure 2,** although mRNA for the variants is expressed prior to castration in the LuCaP 86.2 xenograft xenografts (**ref)** the cells expressing protein for the variant only appear dominant after castration. This is the first time that cells expressing AR-variant proteins have been shown to become the dominant cells in a tumor following emergence from castration.

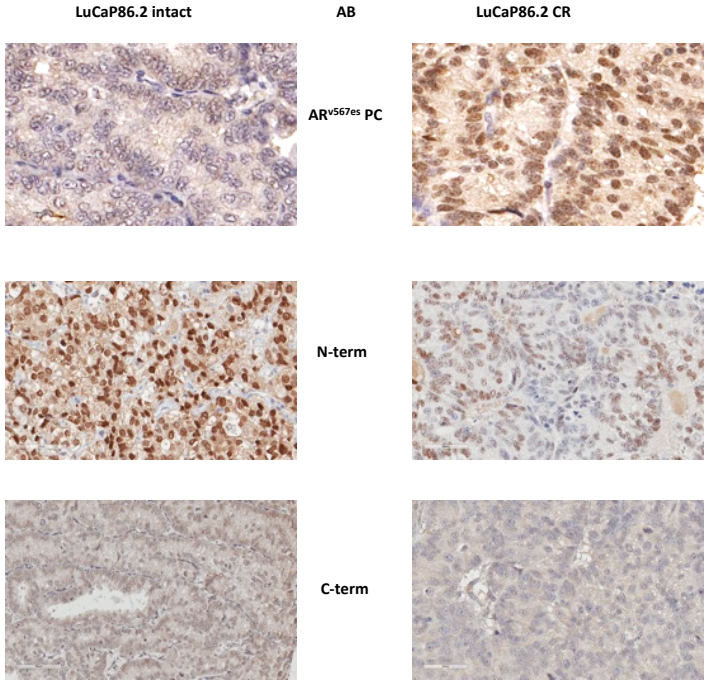


Figure 2. LuCaP 86.2 human prostate cancer xenograft from intact and castrate mice. Note increased AR^{v567es} staining with castration and decreased AR-FL as indicated by C and N-term antibody staining.

Aim 2 will examine the impact of EPI-001 on castration sensitive and castration resistant growth of tumors with differing tumor androgen levels and differing ratios of ARv567es to full-length.

Steroid data. Reported in 2013 annual report.

Aim 3 will elucidate the specific molecular mechanisms by which EPI-001 inhibits the activity of full-length AR and truncated ARv567es variants using in vitro models.

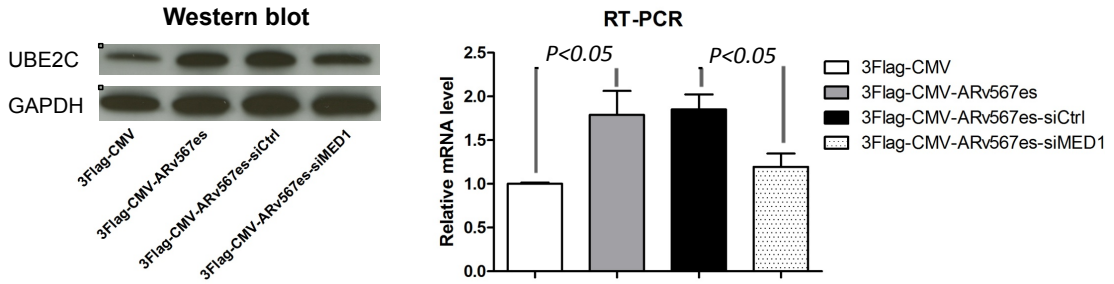


Figure 3. The expression of UBE2C increased in the regular media without DHT addition in LNCaP cells transfected with a lentivirus expressing AR^{v567es}.

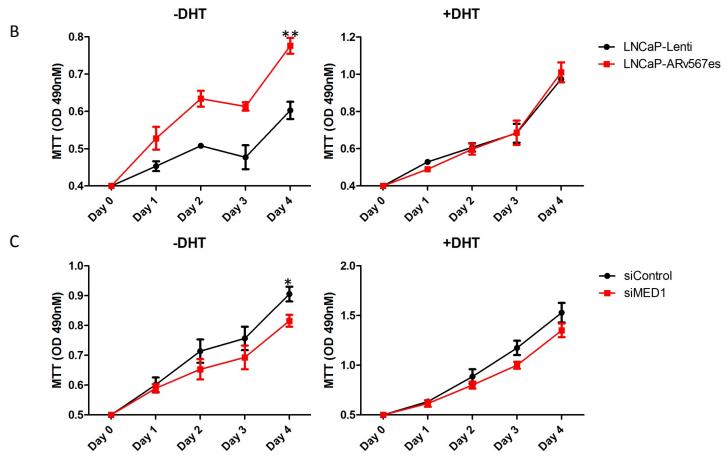


Figure 4. The AR^{v567es} expressing stable cell line LNCaP-AR^{v567es} grows faster than control LNCaP-Lenti cells in the absence, but not in the presence of DHT (Figure 1B). To investigate whether there is a functional interaction between MED1 and AR^{v567es}, we tested the effect of MED1 silencing on AR^{v567es} induced cell proliferation. Silencing of MED1 decreased proliferation of LNCaP-AR^{v567es} cells compared with scramble control (Figure 1C).

During the past funding period we have shown that in the castration state AR-V expressing cells signal through a long range chromatin looping complex using MED 1. This work is demonstrated and explained in **Figures 3-9.**

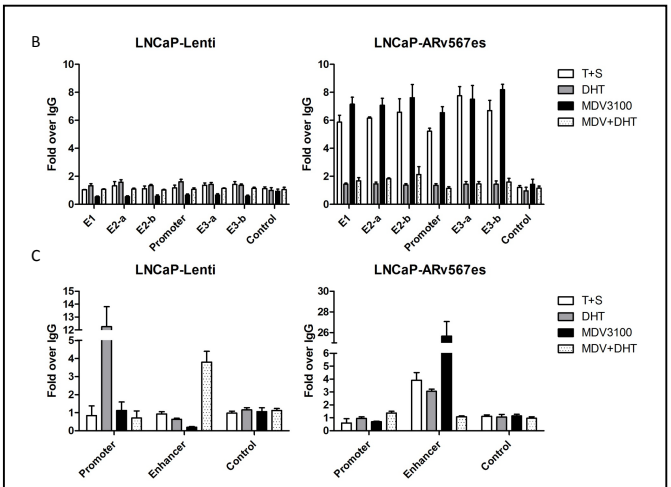
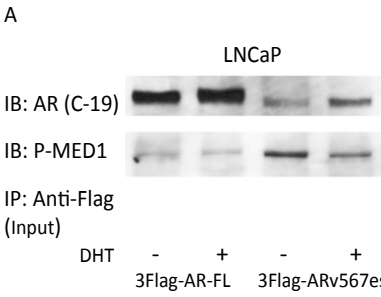


Figure 4. B and C



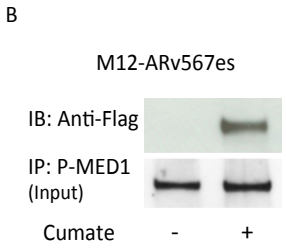


Figure 5. Co-immunoprecipitation assay was performed with the LNCaP cell line transiently transfected with Flag-tagged AR^{v567es}. The anti-Flag antibody could pull down p-MED1as well as AR^{fl}, indicating a physical association of AR^{v567es} with p-MED1 and AR^{fl} in the context of protein activity. Of note, Flag-tagged AR^{fl} also pulled down p-MED1. However, with the same amount of input protein lysates (100 ug), AR^{v567es} showed abundance of p-MED1 co-precipitation especially in the absence of DHT, but AR^{fl} only pulled down much less p-MED1 protein. This finding indicates AR^{v567} has more potency to recruit p-MED1 when androgen is depleted, which is exact in accordance with the impaired cell growth by siMED1 in androgen-depleted condition shown in Figure 2.

Problems- We had been hindered in approach to fully understanding the mechanisms by which EPI compounds work due to a lack of specific antibodies for IP and IHC for AR^{v567es} and AR-V7. As we noted in this report, these antibodies have now been made and are available to our group specifically to use in these studies. This work will be accomplished in the NCE of this proposal.

Progress towards clinic – Finally, although clinical trial support has is not part of this synergy proposal, the work accomplished in this proposal has led to discussions and design to move an EPI compound forward into phase1 clinical trial. IND application is targeted for February 2015.

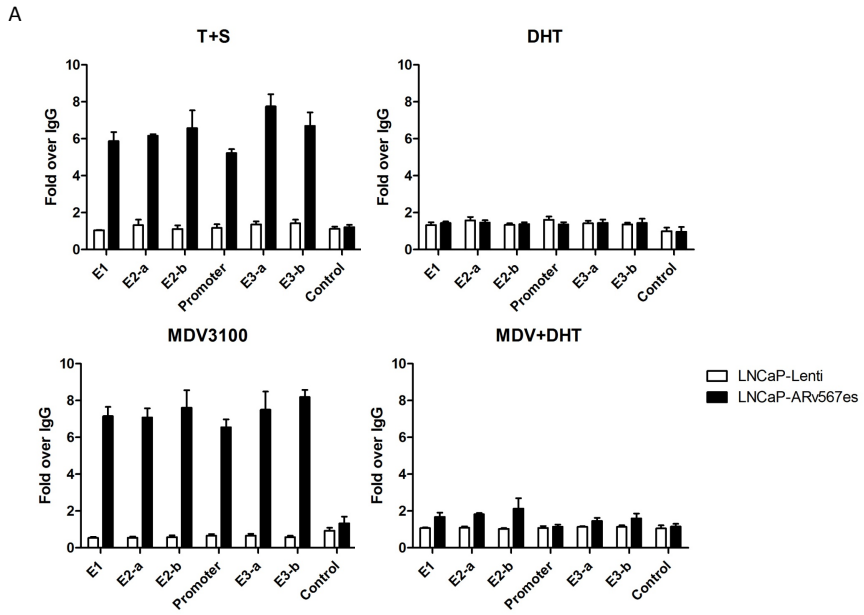


Figure 6.A. Using ChIP assay, we tested p-MED1 binding capacity to the UBE2C promoter and all identified enhancers in LNCaP-Lenti and LNCaP-AR^{v567es} cells under different conditions. More p-MED1 binding (5-8 fold) at UBE2C transcriptional regions, but not at control regions, was seen in LNCaP-AR^{v567es} cell compared to LNCaP-Lenti cell when DHT was absent (T+S media), or when AR^{fl} was inhibited by enzalutamide (MDV3100). However, in the presence of DHT, the increased binding in LNCaP-AR^{v567es} cells diminished to the level seen in the LNCaP-Lenti cells. When both DHT and MDV3100 were present (MDV+DHT), a combination that partially inhibits AR^{fl} activity, the

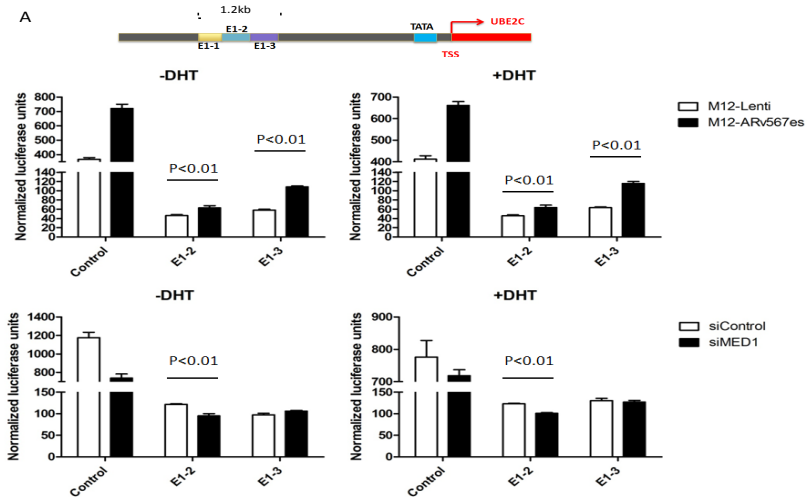


Figure 7. We further studied the role of MED1 on AR^{v567es} induced transcriptional activity in the UBE2C locus. The 1.2kb-long enhancer 1 (E1) fragment was used in a luciferase reporter assay [Figure 5A]. E1 locates 20kb 5' of the UBE2C transcription start site (TSS) and has the highest activity in 3C assay. Three ~400bp regions in Enhancer 1 termed E1-1, E1-2 and E1-3 were systematically subcloned into the pGL4.10-E4TATA-Luc vector. The reporter activities were measured in M12-Lenti cells and M12-AR^{v567es} cells. The M12 prostate cancer cell line is an AR

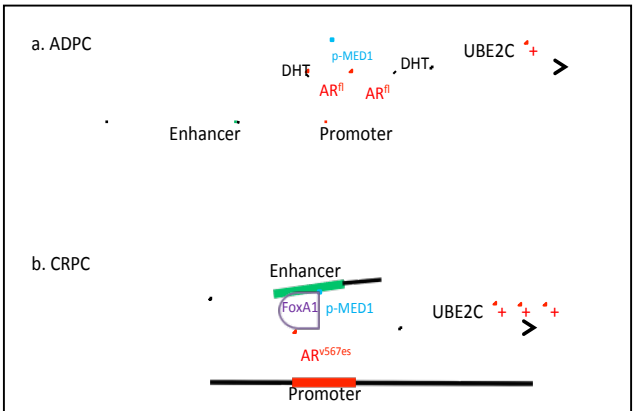


Figure 8. Here we raise a hypothesis (Figure 7) that could reasonably address this: when androgen is available (prior to ADT) in androgen dependent prostate cancer (ADPC), more p-MED1 goes to AR^{fl} but no chromatin looping forms and AR^{fl} has a low level of activation on UBE2C transcription; When AR^{fl} signaling is inhibited in CRPC, AR splice variants are formed due to the stress on the cells as a survival mechanism; the variants have higher affinity to p-MED1, and therefore recruit more p-MED1 to the UBE2C promoter and enhancers with the assistance of FoxA1, resulting in chromatin looping, which strongly enhances UBE2C expression, leading to cell survival and increased cell proliferation.

Key Research Accomplishments

- **AR-SVs generate their unique transcriptome through a long range chromatin looping mechanism.**

Reportable outcomes:
Publications:

1. Liu G, Sprenger C, Sun S, Epilepsia KS, Haugk K, Zhang X, Coleman I, Nelson PS, Plymate S. AR Variant AR(v567es) Induces Carcinogenesis in a Novel Transgenic Mouse Model of Prostate Cancer. Neoplasia. 2013 Sep;15(9):1009-17.PMID:24027426
2. Thadani-Mulero M, Portella L, Sun S, Sung M , Matov A , Vessella RL, Corey E, Nanus DM, Plymate SR, Giannakakou, E. Androgen Receptor Splice Variants Determine Taxane Sensitivity in Prostate Cancer. Cancer Res. 2014 Feb 20. PMID:24556717
3. Cao B, Qi Y, Zhang G, Xu Z, Zhan Y, Alvarez X, Guo Z, Fu X, Plymate SR, Sartor O, Zhang H, Dong Y. Androgen Receptor Splice Variants Activating the Full-Length Receptor in Mediating Resistance to Androgen-Directed Therapy. Oncotarget. 2014 Mar 30;5(6):1646-56.PMID:24722067
4. Sprenger CC, Plymate SR. The Link between Androgen Receptor Splice Variants and Castration Resistant Prostate Horm Cancer. 2014 Aug;5(4):207-17. Epub 2014 May 6.

Conclusion: Thus the results of these studies lead to our further development of the EPI-compounds as important medical products for the treatment of advanced prostate cancer. Scientifically, these studies demonstrate that the N-terminus of the androgen receptor is an important driver of prostate cancer in the absence of ligand. Of particular note this year we have shown that the AR-variants react in a unique way with chromatin to generate the variant transcriptome.

Supporting Data: All data for this report is contained in the Body of the report. No additional supporting data is necessary.